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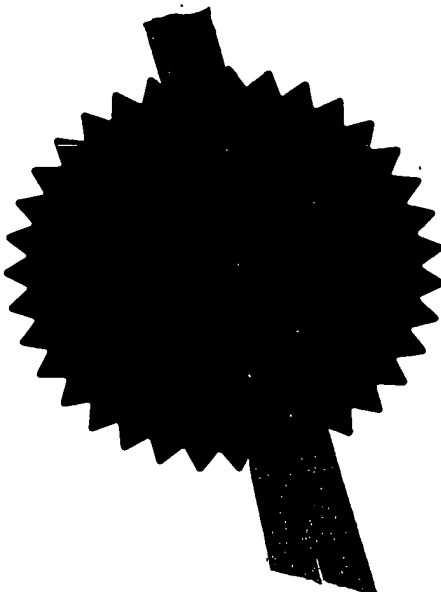
PCT

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*L. Mahoney*

Signed

Dated 26 August 2004



19AUG03 E831314-1 C66582  
P01/7700 0.00-0319422.2

19 AUG 2003

# Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

THE PATENT OFFICE

19 AUG 2003

NEWPORT

The Patent Office

Cardiff Road  
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NP10 8QQ

1. Your reference

CGS 22

2. Patent application number

(The Patent Office will fill this part in)

0319422.2

3. Full name, address and postcode of the or of each applicant (underline all surnames)

CYCLOPS GENOME SCIENCES LIMITED  
14 GUEST ROAD  
CAMBRIDGE CB1 2AL

Patents ADP number (if you know it)

If the applicant is a corporate body, give the country/state of its incorporation

07651862003  
UNITED KINGDOM

4. Title of the invention

CLEAN - UP BEADS

5. Name of your agent (if you have one)

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

CYCLOPS GENOME SCIENCES LIMITED  
14 GUEST ROAD  
CAMBRIDGE CB1 2AL

Patents ADP number (if you know it)

1255003

Page White + Farmer  
54 Doughty St. London  
WC1N 2LS

6. Priority: Complete this section if you are declaring priority from one or more earlier patent applications, filed in the last 12 months.

Country

Priority application number  
(if you know it)

Date of filing  
(day / month / year)

GB

0317199.8

23/07/03

7. Divisionals, etc: Complete this section only if this application is a divisional application or resulted from an entitlement dispute (see note f)

Number of earlier UK application

Date of filing  
(day / month / year)

8. Is a Patents Form 7/77 (Statement of inventorship and of right to grant of a patent) required in support of this request?

Answer YES if:

- a) any applicant named in part 3 is not an inventor, or
- b) there is an inventor who is not named as an applicant, or
- c) any named applicant is a corporate body.

Otherwise answer NO (See note d)

YES

**Patents Form 1/77**

9. Accompanying documents: A patent application must include a description of the invention. Not counting duplicates, please enter the number of pages of each item accompanying this form:

Continuation sheets of this form


Description

17

Claim(s)

Abstract

Drawing(s)



10. If you are also filing any of the following, state how many against each item.

Priority documents

Translations of priority documents

Statement of inventorship and right to grant of a patent (Patents Form 7/77)

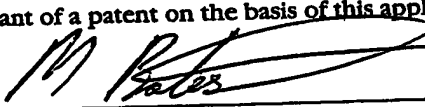
Request for a preliminary examination and search (Patents Form 9/77)

Request for a substantive examination (Patents Form 10/77)

Any other documents (please specify)

11. ☒ We request the grant of a patent on the basis of this application.

Signature(s)



Date 18/08/03

12. Name, daytime telephone number and e-mail address, if any, of person to contact in the United Kingdom

MALCOLM ROBERT BATES 01223 446422

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## **Clean-Up Beads**

Described is a means to differentially separate molecules based on one or more of the molecules' physical properties such as charge and/ or size. Separation occurs by means of a surface treatment of a solid phase where the surface treatment forms a separation between the solid phase and the sample. The solid phase has the capacity to bind one or more of the constituents contained in the sample thereby removing one or more undesired constituents such as a contaminant from the analyte. The solid phase, without any particular surface treatment has the undesirable property to bind both the undesired constituents and the analyte molecules so that no separation occurs. The ability of the solid phase to differentially bind either the analyte or contaminant is dependent on the properties of the surface treatment.

The surface treatment provides a selective barrier for the constituents of the analyte solution to the solid phase. The selectivity is based on the ability of the surface treatment to exclude molecules of a certain size and/or charge from the underlying solid phase. For example, it has surprisingly been found that a polynucleotide surface treatment of hydroxylapatite particles provides a semi-permeable barrier to a mixture of labelled nucleotides and a labelled polynucleotide, whereby the nucleotides are bound by the underlying solid phase because of their relatively small size compared with the polynucleotide. The exclusion of the analyte polynucleotide from the underlying solid phase is based on charge repulsion, binding competition and/or size exclusion by the surface treatment. In this example the unwanted labelled nucleotides can be removed from the desired labelled polynucleotide and the invention provides an efficient means, for example, to remove unincorporated labelled nucleotides from the desired labelled polynucleotide following a labelling reaction (Sambrook et al., (1989) *Molecular Cloning: A Laboratory Manual*, CSH).

Automated high throughput sequencing requires the essential step of removing salts and unincorporated dye labelled terminators from the desired reaction products. Current methods to remove nucleotides from polynucleotides require lengthy and sometimes costly processes, in

particular gel filtration with for example, Sephadex G-10, G-25, G-50 or G-75 (Amersham Biosciences, UK). This method necessitates the pre-swelling of Sephadex beads, pouring a column, replenishment of column buffer and the collection of several fractions in order to determine the fraction containing the desired polynucleotide. Gel filtration of radioactive samples can expose the operator to significant radioactive exposure for substantial periods. Other gel filtration methods make use of spin columns that can be used in centrifuges, which although faster than the use of columns still requires considerable operator hands on time. Gel filtration methods are poorly adapted to automation because they require many centrifugation or vacuum drying steps. Likewise, ethanol precipitation, another commonly used method for the removal, for example, of nucleotides from a polynucleotide is a lengthy and inefficient method that frequently results in substantial sample loss and salt contamination and is not suitable for automation.

A preferred embodiment of the invention is that the undesired constituent such as unincorporated nucleotides are removed and trapped by the solid phase whilst the desired analyte such as DNA remains in solution. Current methods such as silica particle separation of nucleotides and polynucleotides requires the binding of the analyte to the solid phase followed by washing of the particles to remove the undesired constituent, followed finally by elution of the analyte from the solid phase. One of the advantages of this invention is that there is no elution step required because the solid phase binds only those components of the reaction which are non-desired leaving the purified analyte free in solution.

The analyte can be a (i) a polynucleotide such as single or double stranded DNA or RNA such as those derived from a polymerisation reaction such as reverse transcription, PCR, a DNA sequencing reaction, a labelling reaction such as nick translation or random priming, a product of RNA polymerisation such as an *in vitro* transcription reaction (Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual, CSH), (ii) a protein such as an enzyme, a cofactor, a receptor, a glycoprotein, an antibody, a phosphoprotein, an antigen, a diagnostic biomarker or (iii) a small drug like molecule such as a peptide, a steroid or cAMP.

The undesired constituent to be removed can be one or more of the following (i) an unincorporated ribonucleotide, deoxyribonucleotide or dideoxyribonucleotide mono/ di/ or triphosphate such as an unlabelled or fluorescently labelled nucleotide such as Cy5 or Cy3 Cydye™ (Amersham Biosciences, UK), rhodamine, fluorescein, TAMRA, ATTO590, JOE, ROX, coumarin, or biotin, or cholesterol, or aminoallyl labelled or a 3H, 14C, 35S, 33P or 32P radioactively labelled nucleotide mono/ di or triphosphate, (ii) a salt such as CoCl<sub>2</sub>, CaCl<sub>2</sub>, LiCl<sub>2</sub>, MnCl<sub>2</sub>, MgCl<sub>2</sub>, KCl, NaCl or sodium phosphate, (iii) a detergent such as SDS or SLS, (iv) a precursor such as tritium labelled thymidine, a radioactive amino acid such as 35S cysteine or methionine, or 14C glycine, a sugar such as 14C glucose phosphate, or 14C galactose, or phosphorous 32, (iv) a radioactive metal such as chromium 51, calcium 45, cobalt 57, iron 59, (v) a toxic material such as ethidium bromide, (vi) a chelator such as CDTA (trans-1,2-Diaminocyclohexane-N,N,N',N'-tetraacetic acid), EDTA (Ethylenediamine tetraacetic acid), EGTA (Ethyleneglycol-O,O'-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid), DTPA (Diethylenetriamine pentaacetic acid), HEDTA (N-(2-Hydroxyethyl)ethylenediamine-N,N,N'-triacetic acid), NTA (Nitrilotriacetic acid), TTHA (Triethylenetetramine-N,N,N',N'',N''',N''''-hexaacetic acid), Dimethyl-BAPTA (Molecular Probes, USA), citric acid or BAPTA (Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid). Preferably, the undesired constituent bears a positive or negative charge so that it can be bound to the solid phase via a charge attraction. More preferably, the undesired constituent bears a negative charge so that it can be bound and removed using surface modified hydroxylapatite.

The solid phase could be one with a net negative or positive charge, be hydrophobic, have affinity for a molecule for example via an antigen-antibody complex or have size exclusion properties. Examples of suitable solid phases include agarose, acrylamide, polyethylene, polycarbonate, polypropylene, nylon, glass, hydroxylapatite, fluorapatite, silica, metals including; sodium, lithium, potassium, cesium, magnesium, titanium, chromium, manganese, calcium, iron, cobalt, nickel, copper, zinc, aluminium, silver, gold, platinum and lead, a metal oxide, a mixture of metals such as an iron-zinc blend or an oxide thereof and lithium iron III oxide. Preferably the solid phase contains a magnetic component such as iron allowing simplified mixing and separation of the particle from the analyte containing mixture. Magnetic

hydroxylapatite, which is particularly suited to practising this invention, is commercially available (Hydroxylapatite Type I, Chemicell GmbH, Germany).

The solid phase could be in the form of a bead, a particle, a magnetic bead or particle, a sheet, a gel, a powder, a membrane, and be contained within the interior of a tube or as the packing of a chromatography column, or on the lining of a well such as 96, 384 or 1536 formats or on the surface of an elongated probe capable of entering into a vessel. In each case, the solid phase is coated with a surface treatment that provides a means to differentially separate the analyte from the undesired constituent. It could also be a membrane containing pores of a defined size so that the entry of an analyte molecule into the pore is controlled not only by the size of the pore itself but also the charge surrounding and within the pore itself.

The invention therefore relates to the combination of one or more solid phases which may be a chromatography material such as hydroxylapatite with one or more specialised surface treatments thereby restricting the interaction of molecules within the sample with the chromatography material.

The attachment of the surface treatment to the chromatography material is not particularly limited but can be covalent, ionic, encapsulation coating, adsorption, absorption or a hydrophobic interaction. The surface treatment must be sufficiently dense to at least cover, in its entirety the underlying surface phase at a density such that there is useful separation of the undesired component from the analyte. The density and suitability of the surface treatment can be empirically determined by adding less and less of the surface treatment to a fixed amount of solid phase until the desired separation property of a labelled analyte from the undesired component is lost. This can be readily determined by using for example a radioactive or fluorescently labelled analyte and / or undesired constituent. It is preferred that more than 50% of the undesired reaction component is removed from the analyte, more preferably at least 75%, even more preferably at least 90% and most preferably 100%. It is also preferred that less than 50% of the desired analyte is lost during the separation of the undesired constituent, more preferably less than 25%, even more preferably less than 10% and most preferably 0%.

It is preferable that the surface treatment material is sufficiently firmly attached to the underlying solid phase that it does not fall off, leech or in any other way contaminate the analyte containing mixture. The attachment strength can be determined by incubating the surface treated solid phase in the same solvent as the analyte and measuring the loss of the surface treatment from the solid phase by for example weighing, IR spectrophotometry or u/v absorption or loss of the desired separation property from the solid phase. Alternatively, the surface treatment can be labelled with for example fluorescence and the rate of appearance of fluorescence in the analyte containing solvent measured. If the surface treatment is a polynucleotide, it is preferred that it is a non-coding homopolymer chosen from, for example, poly r(C), poly r(U), poly r(G), poly r(A), or poly d(C), poly d(T), poly d(U), poly d(G), poly d(A), or poly d(A/T), poly d(U/A) or poly d(G/C) so that it is less likely to interfere with sensitive downstream applications such as reverse transcription or protein translation. Ribopolynucleotides such as poly r(C) or poly r(A) surface coating can be stabilised from attack by ribonucleases and hydrolysis by modification of the 2'-OH groups with for example acetyl as set out in patent applications WO/01/94626 and WO/00/75302. Ribopolynucleotides can also be covalently cross-linked using glutaldehyde or sebacoyl chloride as set out in patent applications WO/01/94626 and WO/00/75302. The covalent cross-linking produces an interlocked mesh on the exterior of the solid phase so that any one molecule of a polyribonucleotide is less likely escape from the surface of the solid phase. DNA strands can also be cross-linked by for example adriamycin treatment (Cullinane et al, (2000) Nucleic. Acids. Res. 28:1019), mitomycin C, glutaldehyde or u/v cross linking. The cross linking of DNA and RNA is well known in the art and the particular method is not limited, however sufficient cross linking should be obtained to reduce leeching of the surface treatment to a point where it does not interfere with the downstream application and analysis of the analyte.

Polyribonucleotides are sometimes added as enhancers of reverse transcription (HCV Amplicor v2.0, Roche, USA) so the leaching of the surface treatment into the analyte is not necessarily undesired. However, when the downstream use of the analyte is not particularly sensitive to contamination with the surface treatment, for example, the removal of labelled



nucleotides from radioactive probes for Southern blotting, then the type of polynucleotide is not particularly important so that coding polynucleotides, for example genomic DNA such as single or double stranded salmon sperm DNA can be used. Such sources of polynucleotides for surface treatment can be more economical than homopolymers that are produced by an *in vitro* enzymatic or synthetic reaction.

When the surface treatment is a polynucleotide, it is not especially limited by the sequence, save for the reasons expressed above. It can be a single, double or triple stranded homopolymer, a single, double or triple stranded hetero oligo- or polynucleotide such as prokaryotic or eukaryotic genomic DNA such as *E.coli*, plant or salmon or herring sperm genomic DNA, a phage such as Lambda phage, M13, or virus such as BMV or TMV derived nucleic acid, mitochondrial DNA, total RNA, rRNA, tRNA or mRNA. The nucleic acid can be synthesized from an *in vivo* or *in vitro* source such as during an RNA polymerase reaction or PCR. The length of the oligo- or polynucleotide is not particularly limited, however, longer sequences are preferred when used in combination with hydroxylapatite solid phases so that there is an increased chance that there are at least two or more points of charge interaction between the nucleic acid surface treatment and the hydroxylapatite, thereby reducing the amount of the surface treatment that may become unbound from the hydroxylapatite and contaminate the analyte containing solution. Therefore sequences of at least 50 nucleotides, more preferably 250 nucleotides, even more preferably 500 nucleotides and most preferably over 2000 nucleotides in length. The nucleic acid can be either single, double or triple stranded, however, double stranded is preferred because it is an economical source and has a higher affinity for hydroxylapatite than single stranded nucleic acids because of its increased charge. The nucleic acid need not be salt free, for example lithium, potassium, sodium, manganese or magnesium salts of the nucleic acid phosphate groups can be employed and have no special advantages compared with salt-free nucleic acids as surface treatments.

Homopolymers of RNA and DNA, which are particularly preferred for surface treatment of hydroxylapatite, are commercially available as either single or double stranded molecules (Midland Certified Reagent Company, USA and Amersham Biosciences, UK).

The analyte containing mixture can be presented to the separation material either in a liquid such as water, a buffer, a chelator such as EDTA or EGTA, an enzymatic reaction, a cellular or biological fluid such as a cell lysate, serum or cell supernatant used for drug discovery, a homogenised clinical sample, or an organic solvent such as ethanol, DMSO, toluene, tetrahydrofuran or acetonitrile. Alternatively, the mixture containing the analyte and undesired constituent can be in the gas phase or a vapour so that for example a gaseous mixture is pumped through or over the separation mixture consisting of the surface treated solid phase and the purified exhaust gases captured for analysis.

The invention could also be used in combination with another separation process such as filtration, gas chromatography, dialysis or affinity chromatography to render the removal of the undesired constituent in a more precise manner. Indeed the invention is not especially limited as to the type of downstream application that the purified analyte is used for. Such applications include but are not limited to: Southern and Northern blotting; gel electrophoresis of nucleic acids and proteins; electroblotting; cDNA synthesis; PCR amplification; RT-PCR amplification; ligase chain reaction (LCR) amplification; transcription mediated amplification (TMA); single nucleotide polymorphism analysis (SNP); various forms of chromatography such as affinity, ion exchange, hydrophobic interaction, reversed phase and gel filtration; various forms of mass spectrometry (MS) such as, gas chromatography-MS, SELDI, MALDI-TOF, ESI, MS/MS or FT-ICR; capillary electrophoresis particularly of fluorescent dye labelled sequencing reactions and SNP assays using commercial systems such as the ABI Prism 3100, or ABI 3730 (Applied Biosystems, USA) where the removal of unincorporated fluorescent sequencing nucleotides is critical. Indeed, this invention can also be used to remove dNTP's following PCR amplification but preceding the sequencing reaction itself. In this case, it is critical that all the unlabelled nucleotides that were not incorporated into the PCR product during amplification are removed otherwise they will interfere with the sequencing reaction. Currently, unincorporated nucleotide triphosphates are removed following PCR using shrimp alkaline phosphatase (see ExoSAP-IT (Amersham Biosciences; catalogue number US78200). By treating the PCR reaction, post-amplification with the surface treated solid phase as set out

in this invention effectively removes the free nucleotides from the PCR product leading to a successful sequencing reaction. This is particularly well suited to high throughput sequencing and SNP applications.

The invention provides a simple means to remove a labelled molecule from a sample, thereby allowing the quantitation and analysis of the amount of the label that has been metabolized by for example cellular enzymes, or incorporated into a polymer, or broken down (catabolized). This is particularly useful for drug discovery applications for example employing assays to determine the incorporation of <sup>3</sup>H labelled thymidine.

Whilst nucleic acid attachment to a solid phase for purification purposes (for example Immobilised DNA, catalogue number 27-5575-02, and Polynucleotide affinity, catalogue number 17-0860-01, Amersham Biosciences, UK), the purpose of these products is to capture biomolecules such as proteins and RNA on the surface of the solid phase by way of an affinity interaction (Greth et al., (1975) Biochem. Biophys. Acta. 390:168). In contrast, the nature of the present invention is not to bind molecules on its surface, rather it is to selectively stop a proportion of the molecules from binding to the solid phase. Therefore there is no intention to capture molecules by way of the surface treatment rather it is to form a selective barrier to the underlying solid phase.

When the surface treatment is an oligo- or polynucleotide, the surface treatment can provide two different functions; firstly, as set out above, as a selective barrier to the analyte and secondly, as an affinity capture reagent specific for particular nucleotide sequences. For example, if the oligo- or polynucleotide sequence is complementary to a specific sequence such as a human repetitive DNA element then it would be a means to remove not only smaller molecules by means of acting as a selective barrier but also by capturing a sub-group of nucleotide sequences in the analyte mixture. In this way, non-desired nucleic acid sequences can be removed simultaneously with the non-desired molecules such as EGTA, or free nucleotides leaving the purified desired analyte in solution ready to be analysed. For example, when an oligonucleotide or peptide nucleic acid complementary to a human repetitive

sequence such as a Line-1 element is bound on the exterior of magnetic hydroxylapatite beads (Chemicell, Germany) then the surface treated beads are efficient at removing both dNTP's and contaminating human genomic sequences post-PCR.

#### **Preferred method of use**

Hydroxylapatite, also known as hydroxyapatite is a naturally occurring mineral primarily composed of calcium phosphate. It is widely used in the manufacture of a variety of bioimplants and chromatography materials. Chromatography using hydroxylapatite is a well-established method and is principally used for the separation and purification of proteins, DNA and RNA (Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual, CSH).

The interaction is based either on the ionic attraction between a positive charge on the sample molecule and the negative phosphate charge of the hydroxylapatite or a negative charge on the sample molecule and the positive charge of the calcium.

For the hypothetical example of two molecules of identical molecular weight and size but one molecule has no charge and the other a charge of -1. If the surface coating of the membrane is negative, then the uncharged molecule will preferentially pass through the pores of the membrane, whilst the negatively charged molecule will be repulsed and therefore be less likely to enter the pore. The uncharged molecule could be induced to pass through the pore by diffusion, capillarity, pressure or centrifugal force. The uncharged molecule could be detected by a variety of methods including a scintillation proximity assay, a fluorescence assay or colorimetric detection.

#### **Example 1**

##### **Removing unincorporated labelled dideoxynucleotide triphosphates from a DNA sequencing ladder**

Preparation of nucleic acid surfaces on magnetic hydroxylapatite: To 10 $\mu$ l (50mg/ml) of magnetic hydroxylapatite Type-I (Chemicell, Germany) in a 1.5ml polypropylene microcentrifuge tube was added 25 $\mu$ l of water containing 25 $\mu$ g of the homopolymer poly d(A)

(Midland Certified Reagent Company, USA) and mixed for 10 minutes at 25°C using the 0.5 sec. step program of a MCB1200 magnetic mixer (Dexter Magnetics, UK). The beads were collected with the MCB1200 magnet, the liquid removed from the Type-I beads and the beads washed three times with 0.5ml H<sub>2</sub>O each, before finally being resuspended in a final volume of 10µl water. Alternatively, poly d(A) can be replaced with the same amount of poly d(T), poly d(U), poly d(C), poly d(G), poly d(I), poly r(A), poly r(U), poly r(C), poly r(G), poly d(A/T), poly d(C/G), poly d(I/C), poly r(A/U), poly r(A)d(T), poly r(U)d(A), poly r(C)d(G), poly r(G)d(C), BMV RNA (Promega, USA), ssM13 DNA (Amersham Biotech, UK), pUC plasmid DNA, human genomic DNA, single or double stranded salmon or herring sperm DNA (Sigma-Aldrich, USA), 25-50-mer oligonucleotides (MWG, Germany). There is no particular limitation to the type of nucleic acid used except that small amounts of heteropolymers that leach from the magnetic hydroxylapatite such as poly d(I/C) have been found to lead to non-specific PCR amplification products when used to purify templates for PCR amplification. Therefore, for this purpose, non-coding single stranded homopolymers such as poly r(A) are preferred.

## **Example 2**

### **Removing 32P alpha-labelled deoxynucleotide triphosphates from solution**

A comparison was made of four types of nucleic acid coated hydroxylapatite beads prepared as in example 1 in order to determine whether a particular type of nucleic acid provided improved separation properties.

**Preparation of nucleic acid surfaces on magnetic hydroxylapatite:** To 10µl (50mg/ml) of magnetic hydroxylapatite Type-I (Chemicell, Germany) in a 1.5ml polypropylene microcentrifuge tube was added 25µl of water containing 25µg of either the homopolymer poly d(I/C), poly r(A), poly r(C) or poly d(A) (Midland Certified Reagent Company, USA) and mixed for 10 minutes at 25°C using the 0.5 sec. step program of a MCB1200 magnetic mixer. The beads were collected with the MCB1200 magnet, the liquid removed from the Type-I beads and the beads washed three times with 0.5ml H<sub>2</sub>O each, before finally being resuspended in a final volume of 10µl water. The beads were then tested in two separate experiments, for their capacity to bind either free 32P dATP nucleotides or a 32P labelled

mixture of a purified 250 and 1700nt *in vitro* transcribed RNA. To 5µl of the nucleic acid-bead mixture was added either 50µl of water containing  $5 \times 10^3$  cpm  $^{32}\text{P}$  dATP or  $5 \times 10^3$  cpm of RNA. The magnetic beads and radioactive test compound were mixed for 5 minutes at 25°C using a MCB1200 magnetic mixer, before the beads were collected on the side of the tube using a magnet, the liquid removed and both the radioactivity associated with the beads or liquid quantitated by scintillation counting. The results are shown in Table 1, whilst all nucleic acid-hydroxylapatite beads were capable of removing at least 94% of the free nucleotides, poly r(A) was the most effective at preventing the binding and therefore the loss of the RNA analyte.

**Table 1.**

| <b>Nucleic acid surface treatment type</b> | <b>% <math>^{32}\text{P}</math> nucleotides removed from solution</b> | <b>% <math>^{32}\text{P}</math> RNA polynucleotide removed from solution</b> |
|--------------------------------------------|-----------------------------------------------------------------------|------------------------------------------------------------------------------|
| Poly d(I/C)                                | 94                                                                    | 19                                                                           |
| Poly r(C)                                  | 97                                                                    | 14                                                                           |
| Poly r(A)                                  | 95                                                                    | 10                                                                           |
| Poly d(A)                                  | 95                                                                    | 16                                                                           |

### **Example 3**

#### **Determining the minimal amount of nucleic acid required to coat a specified amount of magnetic hydroxylapatite.**

Different amounts of poly d(A) were mixed separately with 10µl (50mg/ml) of magnetic hydroxylapatite Type-I (Chemicell, Germany) and then tested for the capacity of the nucleic acid-bead mixture to bind a  $^{32}\text{P}$  labelled purified mixture of a 250 and 1700nt *in vitro* transcribed RNA in order to determine the minimum required amount.

Preparation of nucleic acid surfaces on magnetic hydroxylapatite: To 10µl (50mg/ml) of magnetic hydroxylapatite Type-I (Chemicell, Germany) in a 1.5ml polypropylene microcentrifuge tube was added 25µl of water containing either 250, 125, 75ng or no poly d(A) (Midland Certified Reagent Company, USA) and mixed for 10 minutes at 25°C using the 0.5 sec. step program of a MCB1200 magnetic mixer (Dexter Magnetics, UK). The beads were

collected with the MCB1200 magnet, the liquid removed from the Type-I beads and the beads washed three times with 0.5ml H<sub>2</sub>O each, before finally being resuspended in a final volume of 10µl water. The results are shown in Table 2.

**Table 2.**

| <b>Amount of poly d(A)</b> | <b>% 32P RNA polynucleotide removed<br/>from solution</b> |
|----------------------------|-----------------------------------------------------------|
| 250ng                      | 9.3                                                       |
| 125ng                      | 7.1                                                       |
| 75ng                       | 30                                                        |
| 0ng                        | 50                                                        |

It was found that between 75-125ng of poly d(A) was required per 10µl magnetic hydroxylapatite Type-I (Chemicell, Germany).

**Example 4**

**Removing unincorporated 32P alpha-labelled deoxynucleotide triphosphates from a  
labelled DNA polymer**

**Example 5**

**Removing unincorporated 32P gamma-labelled deoxynucleotide triphosphates from a  
labelled DNA oligonucleotide**

**Example 6**

**Removing unincorporated dNTP's from a PCR reaction  
Will also remove Pi thereby improving further yields...?**

**Example 7**

**Removing excess salt ions from a sample prior to mass spectrometry analysis**

**Example 8**

**Removing unincorporated  $^{32}\text{P}$  labelled ribonucleotide triphosphates from a labelled RNA polymer**

**Example 9**

**Removing unincorporated  $^{35}\text{S}$  labelled amino acids from a labelled protein**

**Example 10**

**Removing sodium dodecyl sulphate (SDS) from a solution**

SLS and other charged detergents

**Example 11**

**Removing EGTA from a nucleic acid solution**

Preparation of nucleic acid surfaces on magnetic hydroxylapatite: To 10 $\mu\text{l}$  (50mg/ml) of magnetic hydroxylapatite Type-I (Chemicell, Germany) in a 1.5ml polypropylene microcentrifuge tube was added 25 $\mu\text{l}$  of water containing 25 $\mu\text{g}$  of the homopolymer poly r(A) (Midland Certified Reagent Company, USA) and mixed for 10 minutes at 25°C using the 0.5 sec. step program of a MCB1200 magnetic mixer (Dexter Magnetics, UK). The beads were collected with the MCB1200 magnet, the liquid removed and the beads washed three times with 0.5ml H<sub>2</sub>O each, before finally being resuspended in a final volume of 10 $\mu\text{l}$  water. Alternatively, poly r(A) can be replaced with the same amount of poly d(T), poly d(U), poly d(C), poly d(G), poly d(I), poly r(U), poly r(C), poly r(G), poly d(A/T), poly d(C/G), poly d(I/C), poly r(A/U), poly r(A)d(T), poly r(U)d(A), poly r(C)d(G), poly r(G)d(C), 25-50-mer oligonucleotides (MWG, Germany). There is no particular limitation to the type of nucleic acid used except that small amounts of heteropolymers that leach from the magnetic hydroxylapatite such as poly d(I/C) have been found to lead to non-specific PCR amplification products when used to purify templates for PCR amplification. Therefore, for this purpose, non-coding single stranded homopolymers such as poly r(A) are preferred.



Such beads are useful to remove chelators from RNA solutions that have been eluted from magnetic hydroxylapatite as set out in the examples of the patent application GB 0217963.8 (priority date 2<sup>nd</sup> August 2003). In these examples, magnetic hydroxylapatite is used to capture either RNA such as HIV, West Nile Virus or HCV derived from blood or plasma, however, unlike silica, the nucleic acids cannot be eluted using only water and a chelator such as EGTA has been found by the applicant to be effective means to remove the nucleic acid from the magnetic hydroxylapatite. However, for certain downstream applications, in particular RT-PCR using the enzyme Tth DNA polymerase, it has been found that the activity of the enzyme is reduced in the presence of excess chelator. This is because the manganese ions in the reaction that are essential for Tth activity are sequestered by the chelator and this reduces the enzyme activity. It is therefore essential to remove a large proportion of the chelator from the nucleic acid solution if the RNA is to be efficiently copied and amplified using Tth DNA polymerase found for example in the diagnostic kits Ampliscreen and Amplicor HIV and HCV (Roche Diagnostics, USA). Whilst several means to remove the chelator from the nucleic acid solution have been set out by the current applicant in application GB 0217963.8, it has been found that the most effective means is to use nucleic acid coated magnetic hydroxylapatite, and prepared as set out in this example.

The volume of the elution solution containing the chelator is variable and can be within the range of 25-400µl. The preferred elution solution as set out in GB 0217963.8 is 10mM EGTA, pH10.2. Following elution, the residual EGTA concentration is usually reduced to approximately 3-5mM, however there is no particular limitation to the amount of EGTA or other chelator that can be removed from solution; for larger amounts of chelator a correspondingly larger amount of the nucleic acid coated magnetic hydroxylapatite can be used.

Following elution as set out in the examples of GB 0217963.8; to 200µl of the chelator/ nucleic solution (approximate final residual EGTA concentration 5mM EGTA) is added 20µl of the nucleic acid coated magnetic hydroxylapatite prepared as set out in this example using poly r(C) to coat the beads. The nucleic acid coated magnetic hydroxylapatite beads are mixed with

the chelator/ nucleic solution for 10 minutes at 25°C using the 0.5 sec. step program of a MCB1200 magnetic mixer (Dexter Magnetics, UK) during which period, the chelator is bound to the hydroxylapatite underlying the poly r(C) coating but the desired analyte nucleic acid does not bind to the hydroxylapatite because of the poly r(C) coating. The beads are then collected with the MCB1200 magnet and the liquid containing the chelator free nucleic acid, removed from the nucleic acid coated magnetic hydroxylapatite beads and used directly in the assay, containing for example, Tth DNA polymerase. For larger volumes or higher residual concentrations chelator in the elution solution, a correspondingly larger amount of the nucleic acid coated magnetic hydroxylapatite can be used.

The chelator can be any one of several that has affinity for hydroxylapatite, for example; CDTA (trans-1,2-Diaminocyclohexane-N,N,N',N'-tetraacetic acid), EDTA (Ethylenediamine tetraacetic acid), EGTA (Ethyleneglycol-O,O'-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid), DTPA (Diethylenetriamine pentaacetic acid), HEDTA (N-(2-Hydroxyethyl)ethylenediamine-N,N,N'-triacetic acid), NTA (Nitrilotriacetic acid), TTHA (Triethylenetetramine-N,N,N',N'',N''',N''''-hexaacetic acid), Dimethyl-BAPTA (Molecular Probes, USA) or BAPTA (Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid).

The desired nucleic acid in the elution can be RNA, DNA, a hybrid of RNA and DNA, single or double stranded, linear or circular and be derived from a plant, an animal, a bacteria, a virus, a diagnostic sample such as blood or body fluid. The desired nucleic acid can be viral RNA, tRNA, rRNA, mRNA, hnRNA, an aptamer, a plasmid, an oligonucleotide, a polynucleotide, genomic DNA, viral DNA or a ribozyme.

## **Example 12**

### **Removing a chelator from a solution**

CDTA (trans-1,2-Diaminocyclohexane-N,N,N',N'-tetraacetic acid), EDTA (Ethylenediamine tetraacetic acid), EGTA (Ethyleneglycol-O,O'-bis(2-aminoethyl)-N,N,N',N'-tetraacetic acid), DTPA (Diethylenetriamine pentaacetic acid), HEDTA (N-(2-Hydroxyethyl)ethylenediamine-

N,N,N'-triacetic acid), NTA (Nitrilotriacetic acid), TTHA (Triethylenetetramine-N,N,N',N'',N''',N''''-hexaacetic acid), Dimethyl-BAPTA (Molecular Probes, USA) or BAPTA (Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid).

**Example 13**

**Removing sodium phosphate from a polymer eluted from HPA**

**Example 14**

**Removing EtBr from a polymer**

EtBr will bind both HPA and fur on beads.

**Example 15**

**Acetylated RNA**

RNase resistant

**Example 16**

**Immobilising polymer on HPA surface by cross-linking**

Network.

**Example 17**

**Removing salt from a solution**

List salts

**Example 18**

**Removing fluorescently labelled nucleotides from a fluorescently labelled probe for  
microarray analysis**

DNA or RNA

**Example 19**

**Removing biotin labelled nucleotides from a biotinylated probe**

DNA or RNA surface treatment of SA beads (Dynal)

**Example 20**

**Removing fluorescently labelled nucleotides from a fluorescently labelled probe for SNP genotyping studies**

**Example 21**

**Removing biotin labelled nucleotides from a biotinylated probe**

DNA or RNA surface treatment of SA beads (Dynal)

Problem after PCR to sequence due to excess unlabelled nucleotides